FEDERAL REPUBLIC OF GERMANY

CERTIFICATION

The Gesellschaft für Biotechnologische Forschung mbH (GBF) in Braunschweig/Germany has filed a patent application under the title

"EPOTHILONS C AND D, SYNTHESIS AND AGENTS"

on November 18, 1996 with the German Patent Office.

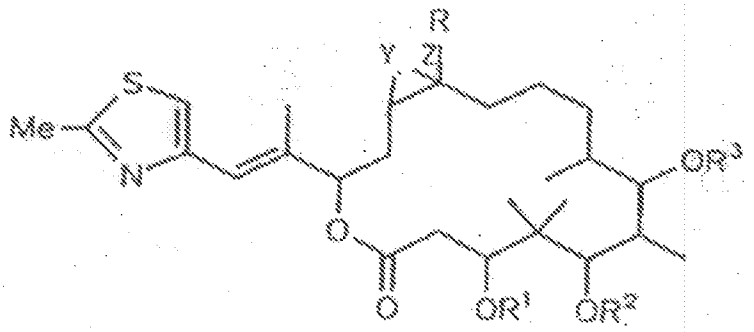
The appended pieces are a correct and accurate reproduction of the original documents of this patent application.

In the German Patent and Trademark Office, the application has received the provisional symbol C 07 D, C 07 F and A 61 K of the International Patent Classification.

Munich, August 17, 1999
The President of the German Patent and Trademark Office
per Wehner

File No.: 196 47 580.5

GBF EXHIBIT 2004
GBF v. Sloan-Kettering Inst.
Contested Case 105,298



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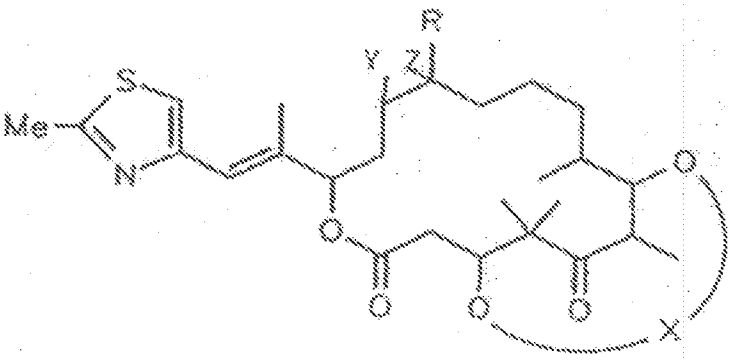

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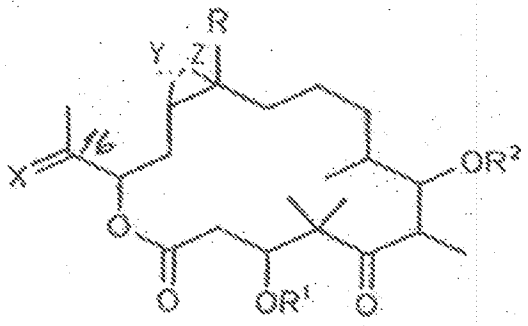

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In the above Formulas 1 to 7,

R = H, C_{1-4} alkyl;

R^1, R^2, R^3, R^4, R^5 = H, C_{1-6} alkyl,
C_{1-6} acyl – benzoyl,
C_{1-4} trialkylsilyl,
benzyl,
phenyl
C_{1-6} alkoxy
C_6 alkyl, hydroxy and halogen-substituted benzyl or phenyl,

two of the R^1 and R^5 groups also being able to come together to form the $-(CH_2)_n-$ grouping with n = 1 to 6 and the alkyl or acyl moieties, contained in the groups, being linear or branched groups,

Y and Z are either the same or different and, in each case, represent hydrogen, halogen, such as fluorine, chlorine, bromine or iodine, pseudo-halogen, such as –NCO, -NCS or $-N_3$, -OH, O-(C_{1-6})-acyl, O-(C_{1-6})-alkyl, O-benzoyl. Y and Z may also be the oxygen atom of an epoxide, epothilon A and B not being claimed, or form one of the C-C bonds of a C=C double bond.

In Formula 3, X generally represents –C(O)-, -C(S)-, -S(O)-, CR^1R^2-, R^1 and R^2 having a same meaning as above, and $-SiR^2$-, in which R has the meaning given above.

In Formula 4, X represents oxygen, NOR^3, N-NR^4R^5 and N-$NHCONR^4R^5$, the R^3 to R^5 groups having the meanings given above.

In Formula 5, X represents hydrogen, C_{1-18} alkyl, C_{1-18} acyl, benzyl, benzoyl and cinnamoyl.

subsequently maybe converted by standard procedures, known to those of average skilled in the art, to oximes, hydrazones or semicarbazones. Furthermore, they are converted into C-16/C-17 olefins by the Wittig, Wittig-Horner, Julia or Petersen olefination.

By reducing the C-16 keto group, for example, with an aluminum or boron hydride, the 16-hydroxy derivatives of the general Formula 5 may be obtained. These can be acylated or alkylated selectively if the 3-OH and the 7-OH groups are provided with appropriate protective groups. The 3-OH and 7-OH groups are freed, for example, by NH_3/methanol in the case of O-formyl and by DDQ in the case of O-p-methoxybenzyl.

The compounds of the general Formula 6 are obtained from the derivatives of epothilon A and B, for which the 7-OH groups. is protected by acyl or ether groups, in that the 3-OH group is, for example, formylated, mesylated or tosylated and subsequently eliminated by treatment with a base, such as DBU. The 7-OH group can then be liberated as described above.

Compounds of the general Formula 7 are obtained by basic hydrolysis, for example with sodium hydroxide in methanol or methanol/water, from epothilon A and B or their derivatives, in which the 3-OH and 7-OH groups are protected. Preferably, compounds of the general Formula 7 are obtained by enzymatic hydrolysis, especially with esterases or lipases, from epothilon A or B or their derivatives, in which the 3-OH or 7-OH group is protected. After protection of the 19-OH group, the carboxyl group can be converted into an ester group by alkylation with diazoalkanes.

Furthermore, compounds of Formula 7 can be converted by lactonization using the methods of Yamaguchi (trichlorobenzoyl chloride/DMAP), Corey (aldrithiol/triphenyl phosphine) or Kellogg (omega hydrogen bromide/cesium

Examples

Example 1:

Compound 1a

Epothilon A (20 mg, 0.041 mmoles) is dissolved in 1 mL of acetone, mixed with 50 µL (0.649 mmoles) of trifluoroacetic acid and stirred overnight at 50°C. For the working up, the reaction mixture is mixed with 1M phosphate buffer of pH 7 and the aqueous phase is extracted four times with ethyl acetate. The combined organic phases are washed with saturated sodium chloride solution, dried over sodium sulfate and freed from solvent. The crude product is purified with the help of preparative layer, chromatography (solvent: 85 : 15 dichloromethane/acetone).

Yield: 4 mg (19%) of isomer I
4 mg (19%) of isomer II

Isomer I

R_f (85 : 15 dichloromethane/acetone): 0.46

IR (Film): $\nu\tilde{}$ = 3440 (m, b, Sch), 2946 (s, Sch), 1734 (vs), 1606 (m), 1456 (m), 1375 (w), 1256 (s, Sch), 1190 (w, b, Sch), 1071 (m, Sch), 884 (w), 735 (w) cm^{-1}.

MS (20/70 eV): m/e (%) = 493 (43 $(M-H_2O)^+$), 394 (47), 306 (32), 206 (30), 181 (40), 166 (72), 139 (100), 113 (19), 71 (19), 57 (24), 43 (24).

High Resolution: ($C_{26}H_{39}O_6NS$ calc.: 493.2498 for $(M-H_2O)^+$
Found: 493.2478

IR (Film): ny = 3441 (s, br, Sch), 2948 (s, Sch), 1725 (vs, Sch), 1462 (m), 1381 (w), 1265 (m), 1154 (w), 972 (m, br, Sch) cm^{-1}.

UV (Methanol): $lambda_{max}$ (lg epsilon) = 210 (4.29), 248 (4.11) nm.

MS (20/70 eV): m/e (%) = 529 (13 [M^+]), 494 (10), 342 (38), 306 (23), 198 (32), 164 (100), 140 (31), 113 (15), 57 (16).

High-resolution: $C_{26}H_{40}O_6ClNS$ calc.: 529.2265 for (M^+), found: 529.2280

Example 3:

Compound 1c

12-Chloro-13-hydroxy-epothilon A (1b) (25 mg, 0.047 mmoles) is dissolved in 1 mL of dichloromethane, mixed with 29 mg (0.235 mmoles) of dimethylaminopyridine, 151 µL (1.081 mmoles) of triethylamine and 20 µL (0.517 mmoles) of 98% formic acid. The reaction mixture is cooled in a mixture of salt and ice. Upon reaching a temperature of -15°C, 40 µL (0.423 mmoles) of acetic anhydride are added to the reaction mixture and stirring is continued for 70 minutes at -15°C. Since a thin-layer chromatogram indicated that the reaction was not completed, a further 6 mg (0.047 mmoles) of dimethylaminopyridine, 7 µL (0.047 mmoles) of triethylamine, 2 µL of 98% formic acid (0.047 mmoles) of acetic anhydride were added to the reaction mixture and stirring was continued for 60 minutes. For the working up, the reaction mixture is heated to room temperature and mixed with 1M phosphate buffer of pH 7 and the aqueous phase is extracted four times with ethyl acetate. The combined organic phases are washed with saturated sodium chloride solution, dried over sodium sulfate and freed from solvent. The crude product is purified with the help of preparative layer chromatography (solvent: 90 : 10 dichloromethane/acetone). Yield: 5 mg (18%).

Example 5:

Compound 2a:

Epothilon A (100 mg, 0.203 mmoles) is dissolved in 4 mL of 1 : 1 tetrahydrofuran/1M phosphate buffer of pH 7 and treated with sodium borohydride (150 mg = 3.965 mmoles) until the educt, according to thin-layer chromatography, has reacted completely. Subsequently, the reaction mixture is diluted with 1M phosphate buffer of pH 7 and the aqueous phase is extracted four times with ethyl acetate. The combined organic phases are washed with saturated sodium chloride solution, dried over sodium sulfate and freed from solvent. The crude product is purified by siliceous chromatography (solvent: 95 : 5 dichloromethane/acetone → grad after 85 : 15 dichloromethane/acetone.

Yield: (20%)

R_f (75: 25 dichloromethane/acetone): 0.27

IR (Film): ny = 3413 (s, b, Sch), 2965 (vs, Sch), 1734 (vs), 1458 (m, b, Sch), 1383 (m, Sch), 1264 (s, b, Sch), 1184 (m, b, Sch), 1059 (s, Sch), 966 (s), 885 (w), 737 (m) cm^{-1}

MS (20/70 eV): m/e (%) = 495 (6 [M^+]), 477 (8), 452 (12), 394 (9), 364 (16), 306 (49), 194 (19), 178 (35), 164 (100), 140 (40), 83 (21), 55 (27).

High-resolution: $C_{26}H_{41}O_6NS$ calc.: 495.2655 for (M^+)

Found: 495.2623

R_f (90: 10 toluene/methanol): 0.44

IR (Film): ny = 2963 (s, br, Sch), 1740 (vs), 1703 (s),, 1510 (w), 1464 (m, br, Sch), 1389 (m, Sch), 1240 (s, br; Sch), 1142 (m), 1076 (w), 1037 (w), 1003 (m), 945 (s, br, Sch), 806 (m, Sch), 775 (s), 737 (m) cm^{-1}.

UV (Methanol): $lambda_{max}$ (lg epsilon) = 211 (4.16), 250 (4.00) nm.

MS (20/70 eV): m/e (%) = 539 (27 (M^+)), 475 (17), 322 (41), 306 (67), 222 (16), 206 (17), 194 (19), 178 (32), 164 (100), 151 (33), 125 (18), 113 (15), 96 (39), 81 (23), 64 (58), 57 (42), 41 (19).

High-resolution: $C_{26}H_{37}O_7NS_2$ calc.: 539.2011 for (M^+)

Found: 539.1998

Compound 3c:

Yield: 4 mg (4%)

R_f (90: 10 toluene/methanol): 0.38

MS (20/70 eV): m/e (%) = 539 (51 (M^+)), 322 (22), 306 (63), 222 (36), 178 (31), 164 (100), 151 (41), 96 (25), 81 (20), 69 (26), 55 (25), 41 (25).

High-resolution: $C_{26}H_{37}O_7NS_2$ calc.: 539.2011 for (M^+)

Found: 539.2001

Compound 3d

Yield: 1 mg (1%)

R_f (90: 10 dichloromethane/acetone): 0.33

MS (20/70 eV): m/e (%) = 539 (69 (M^+)), 322 (35), 306 (53), 222 (41), 178 (31), 164 (100), 151 (46), 96 (31), 81 (26), 69 (34), 55 (33), 41 (35)

Example 8:

Compound 6a

3,7-Di-O-formyl-epothilon A (10 mg, 0.018 mmoles) is dissolved in 1 mL of dichloromethane, treated with 27 µL (0.180 mmoles) of 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) and stirred for 60 minutes at room temperature. The reaction mixture is worked up with 1M sodium dihydrogen phosphate buffer of pH 4 .5 and the aqueous phase is extracted four times with ethyl acetate. The combined organic phases are washed with saturated sodium chloride solution, dried over sodium sulfate and freed from solvent. After removal of the solvent, the resulting crude product is dissolved in 1 mL of methanol, treated with 200 µL of ammoniacal methanol solution (2 mmoles of NH_3/mL of methanol) and stirred overnight at room temperature. The product is worked up by removing the solvent under vacuum.

Yield: 4 mg (22%)

R_f (85: 15 dichloromethane/acetone): 0.46

IR (KBr): ny = 3445 (w, br, Sch), 2950 (vs, br, Sch), 1717 (vs, Sch), 1644 (w), 1466 (m, Sch), 1370 (m, SCh), 1267 (s, br, Sch), 1179 (s, Sch), 984 (s, Sch), 860 (w), 733 (m) cm^{-1}.

UV (Methanol): $lambda_{max}$ (lg epsilon) = 210 (4.16) nm.

MS (20/70 eV): m/e (%) = 475 (28 [M^+]), 380 (21), 322 (37), 318 (40), 304 (66), 170 (31), 166 (100), 151 (29), 140 (19), 96 (38), 81 (20), 57 (26).

High-resolution: $C_{25}H_{37}O_5NS$ calc.: 475.2392 for (M^+)

Found: 475.2384

Example 10:

Compound 6c

3,7-Di-O-acetyl-epothilon (5 mg, 0.009 mmoles) is dissolved in 1 mL of methanol, treated with 150 µL of an ammoniacal methanol solution (2 mmoles NH_3/mL of methanol) and stirred overnight at 50°C. The product is worked up by removing the solvent under vacuum. The crude product is purified with the help of preparative layer chromatography (solvent: 90 : 10 charting/methanol).

Yield: 3 mg (67%)

R_f (90: 10 dichloromethane/acetone): 0.55

IR (Film): ny = 2934 (s, b, Sch), 1719 (vs, b, Sch), 1641 (m), 1460 (m, Sch), 1372 (s, Sch), 1237 (vs, b, Sch), 1179 (s, Sch), 1020 (s), 963 (s, Sch), 737 (vs) cm^{-1}.

UV (Methanol): $lambda_{max}$ (lg epsilon) = 210 (4.33) nm.

MS (20/70 eV): m/e (%) = 517 (57 [M^+]), 422 (58), 318 (31), 194 (20), 181 (34), 166 (100), 151 (31), 96 (96), 81 (32), 69 (27), 55 (29), 43 (69).

High-resolution: $C_{28}H_{39}O_6NS$ calc.: 517.2498 for (M^+)

Found: 517.2492

Example 11:

Compound 7a:

Epothilon (20 mg, 0.041 mmoles) is dissolved in 0.5 mL of methanol, treated with 0.5 mL of 1N sodium hydroxide solution and stirred for five minutes at room temperature. The reaction mixture is worked up with 1M phosphate buffer of pH 7 and the aqueous phase is extracted four times with ethyl acetate. The combined organic phases are washed with saturated sodium chloride solution, dried over sodium

MS (20/70 eV): m/e (%) = 567 (1 [M^+]), 465 (4), 422 (7), 388 (5), 194 (8), 182 (7), 168 (65), 164 (17), 140 (100), 97 (10), 71 (22), 43 (27).

High-resolution: $C_{27}H_{45}O_5NS$ calc.: 567.2866 for (M^+)

Found: 567.2849

Example 13:

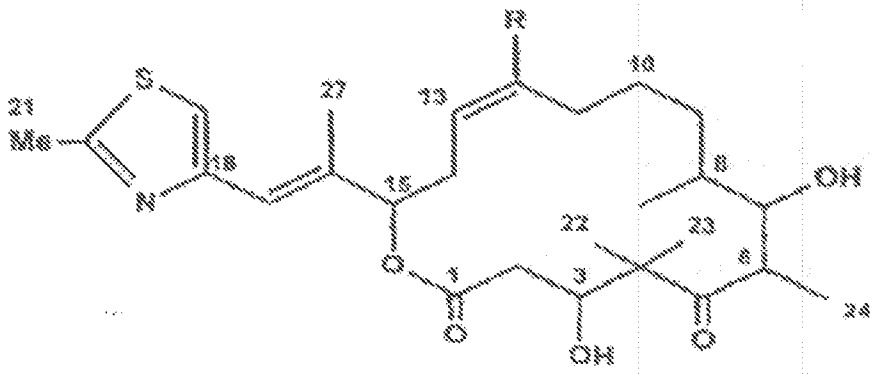
Epothilon A (50 mg) is dissolved in 20 µl of dimethyl sulfoxide and diluted with 30 mL of phosphate buffer (pH of 7.1, 30mM). After the addition of 5 mg of pig's liver esterase (Boehringer Mannheim), the solution is stirred for 2 days at 30°C. After acidification with 2N HCl to a pH of 5, the epothilon acid 7 is extracted with ethyl acetate. The organic phase is dried with sodium sulfate, evaporated to dryness under vacuum. Yield 48 mg (96%).

Example 14:

Epothilon acid 7 (48 mg) is dissolved in 6 mL of THF abs. and treated, while stirring, with 40 µL of triethylamine and 16 µL of 2,4,6-trichlorobenzoyl chloride. After 15 minutes, the precipitate is removed by filtration and the solution is added dropwise of a period of 15 minutes, with rapid stirring, to a boiling solution of 20 mg of 4-dimethylaminopyridine in 200 mL of toluene abs.. After a further 10 minutes, the solution is evaporated under vacuum and the residue distributed between ethyl acetate and citrate buffer of pH 4. The residue, after evaporation of the organic phase and preparative HPLC separation, yields 15 mg of epothilon A.

After epothilon A and B, epothilon C is eluted with a retention time of 90-95 minutes and epothilon D is eluted with a retention time of 100-110 minutes and, after evaporation under vacuum, are obtained as colorless oils in a yield of, in each case, 0.3 g.

D. Physical Properties



Epothilon C R = H

Epothilon D R = CH_3

Epothilon C

$C_{26}H_{39}NO_5S$ [477]

ESI-MS (positive ions): 478.5 for $[M+H]^+$

1H and 13C see NMR Table

TLC: R_f = 0.82

TLC aluminum foil 60 F 254 Merck, solvent: 9 : 1 dichloromethane/methanol

Detection: UV extinction at 254 nm. Spraying with vanillin/sulfuric acid reagent, blue-grey coloration upon heating to 120°C

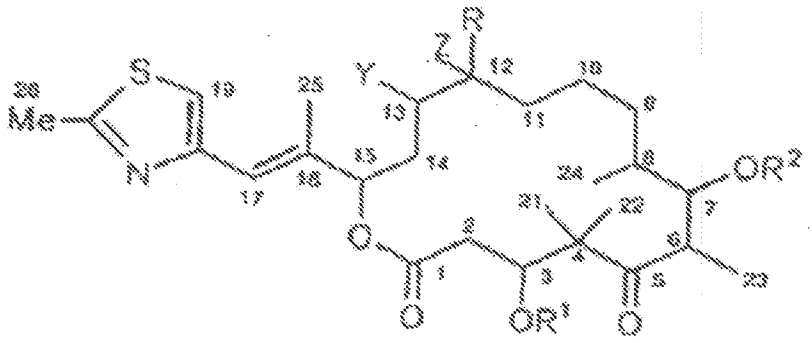
Table: ^{1}H and ^{13C} NMR data of epothilon C and epothilon D in [D_6] DMSO at 300 MHz

	Epothilon C			Epothilon D		
H-Atom	δ (ppm)	C-Atom	δ (ppm)	δ (ppm)	C-Atom	δ (ppm)
		1	170.3		1	170.1
2-Ha	2.38	2	38.4	2.35	2	39.0
2-Hb	2.50	3	71.2	2.38	3	70.8
3-H	3.97	4	53.1	4.10	4	53.2
3-OH	5.12	5	217.1	5.08	5	217.4
6-H	3.07	6	48.4	3.11	6	44.6
7-H	3.49	7	75.9	3.48	7	75.5
7-OH	4.46	8	35.4	4.46	8	36.3
8-H	1.34	9	27.6	1.29	9	29.3
9-Ha	1.15	10	30.0	1.14	10	28.9
9-Hb	1.40	11	27.6	1.38	11	31.0*
10-Ha	1.15*	12	124.6	1.14*	12	138.3
10-Hb	1.35*	13	133.1	1.35*	13	120.3
11-Ha	1.90	14	31.1	1.75	14	31.6*
11-Hb	2.10	15	76.3	2.10	15	76.8
12-H	5.36**	16	137.3		16	137.2
13-H	5.44**	17	119.1	5.08	17	119.2
14-Ha	2.35	18	152.1	2.30	18	152.3
14-Hb	2.70	19	117.7	2.65	19	117.7
15-H	6.27	20	164.2	6.29	20	164.3
17-H	6.50	21	18.8	6.51	21	18.9
18-H	7.35	22	20.0	7.35	22	19.7
21-H_3	2.65	23	22.6	2.65	23	22.5
22-H_3	0.94	24	16.7	0.90	24	16.4
23-H_3	1.01	25	18.4	1.19	25	18.4
24-H_3	1.06	27	14.2	1.07	26	22.9
25-H_3	0.89			0.91	27	14.1
26-H_3				1.63		
27-H_3	2.10			2.11		

*, ** assignment interchangeable

Claims

1. Epothilon derivative of Formula I

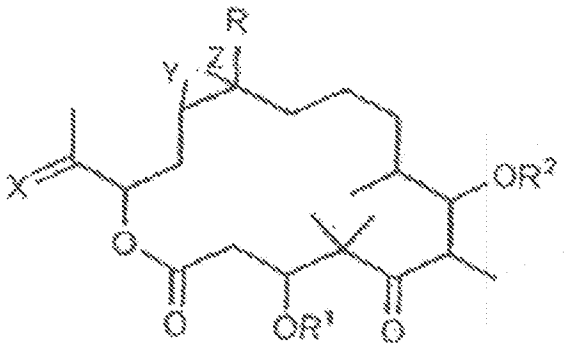


I

in which R = H, C_{1-4} alkyl, R^1, R^2 = H, C_{1-6} alkyl, C_{1-6} acyl, benzoyl, C_{1-4} trialkylsilyl, benzyl, phenyl, benzyl or phenyl substituted by C_{1-6} alkoxy, C_6 alkyl, hydroxy and halogen, the alkyl and acyl moieties contained in the groups, being linear or branched groups and Y and Z being identical or different and in each case representing hydrogen, halogen, pseudohalogen, OH, O-(C_{1-6}) alkyl, O-(C_{1-6}) alkyl or O-benzoyl or jointly forming the O atom of an epoxide or one of the C-C bonds of a C=C double bond, epothilon A and epothilon B being excluded.

in which R = H, C_{1-4} alkyl, R^1, R^2 = H, C_{1-6} alkyl, C_{1-6} acyl, benzoyl, C_{1-4} trialkylsilyl, benzyl, phenyl, benzyl or phenyl substituted by C_{1-6} alkoxy, C_6 alkyl, hydroxy and halogen, the alkyl and acyl moieties contained in the groups, being linear or branched groups and X generally representing –C(O)-, –C(S)-, –S(O)-, $-CR^1R^2$- and $-SiR_2$, R, R^1 and R^2 having the meaning given above and R^1 and R^2 also together being able to form an alkylene group with 2 to 6 carbon atoms and Y and Z having the meanings of claim 1.

4. The Epothilon derivative of Formula 4



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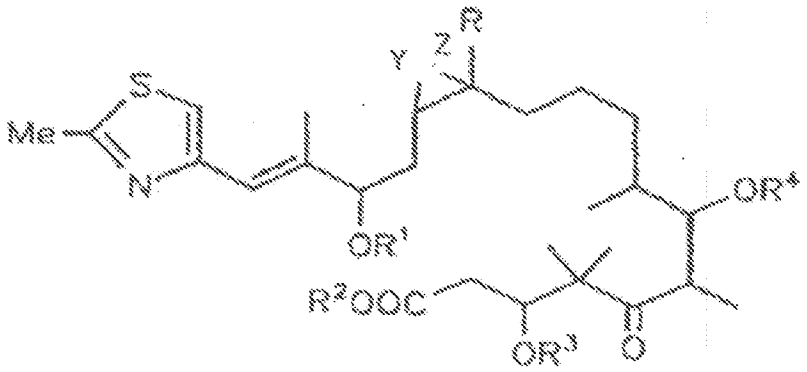
in which R = H, C_{1-4} alkyl, R^1, R^2, R^3, R^4, R^5, = H, C_{1-6} alkyl, C_{1-8} acyl, benzoyl, C_{1-4} trialkylsilyl, benzyl, phenyl, benzyl or phenyl substituted by C_{1-6} alkoxy, C_6 alkyl, hydroxy and halogen, the alkyl and acyl moieties contained in the groups, being linear or branched groups, X represents oxygen, NOR^3, $N\text{-}NR^4R^5$ and$N\text{-}NHCONR^4R^5$, the R^3 to R^5 groups having the meaning given above and R^4 and R^5 also together being able to form an alkylene group with 2 to 6 carbon atoms and Y and Z having the meanings of claim 1

in which R = H, C_{1-4} alkyl, R^1 = H, C_{1-6} alkyl, C_{1-6} acyl, benzoyl, C_{1-4} trialkylsilyl, benzyl, phenyl, benzyl or phenyl substituted by C_{1-6} alkoxy, C_6 alkyl, hydroxy and halogen, the alkyl and acyl moieties contained in the groups, being linear or branched groups and Y and Z having the meanings of claim 1.

7. The epothilon derivative of Formula 7



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in which R = H, C_{1-4} alkyl, R^1, R^2, R^3, R^4 = H, C_{1-6} alkyl, C_{1-6} acyl, benzoyl, C_{1-4} trialkylsilyl, benzyl, phenyl, benzyl or phenyl substituted by C_{1-6} alkoxy, C_6 alkyl, hydroxy and halogen, the alkyl and acyl moieties contained in the groups, being linear or branched groups and Y and Z having the meanings of claim 1.

8. Method for synthesizing an epothilon derivative of Formula 7 of claim 7, characterized in that epothilon A, epothilon B, a derivative thereof protected at the 3 OH or a derivative thereof protected at the 7OH

(a) is hydrolyzed enzymatically, especially with an esterase or a lipase, or

(b) is hydrolyzed in an alkaline medium, especially with sodium hydroxide in a mixture of methanol and water,

and the epothilon derivative of Formula 7 is obtained and isolated.